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CURRENT AND FUTURE DEVELOPMENTS IN CLONAL GERMPLASM CONSERVATION OF VEGETATIVELY PROPAGATED CROPS

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INTRODUCTION

Plant genetic resources are rapidly erod or vanishing due to deprivation of habitats and selective reduction, leading to genetic vulnerability endangering the plant industry and future needs (Frankel and Soule 1981). The conservation of germplasm becomes an urgent necessity, asit plays a crucial role in the improvement of crop variety and food production. The seeds or pollens could serve as functional vector carrying or transmitting genetic information and readily preserved as representive of species genome or genetic diversity. But majority of perennial crops such as fruits, nuts, some tuberous crops and ornamental trees are propagated vegetatively. Ten of these crops like potato, sweet potato, cassava, grape, sugarcane, orange, banana, apple, yam and mango were ranked within the world's top 30 crops (Ford-Lloyd 1990). Asexually propagated crops are characterized by long juvenility and highly genetic heterozygosity, which are not easily duplicated by seeds. Most of tropical and subtropical fruits, large seeded trees and economical importment crops as palm, cocoa and rubber are considered as recalcitrant seeds which are sensitive to dessiccation and chilling (Hartmann et al, 1991), the short longevity becomes unsuitable for long-term seed storage. Additionally, the sexual infertility exists in triploid bananas--its genetic variability mainly derived from somatic mutation, and the double-flowers crops and some breeding lines are difficult to produce seeds. The germplasm maintenance of sterile crops are dependent on vegetative propagules.

The strategy of germplasm conservation depends on the nature of the material and on the objective and scope of the activity (Ford-Lloyd

and Jackson 1986; Towill 1988). For practical purposes in production of uniform clones and due to the limitation of seed problem, the asexually propagated crops are used to place vegetative parts or plant form in a repository maintained *in situ* and *ex situ*. Their contributions and disadventages would be discussed. Basic efforts on long-term conservation of genetic resource are to maximize the diversity and prevent genetic erosion, to minimize maintenance cost and to enhance utilization efficiency. In reality, the conservation of each crop in a clonal genebank is much expensive and less efficiency than in a seed genebank. Considering economy of scale and efficiency, alternative method of *in vitro* conservation of tissues or DNA have been developed. The application of tissue culture for clonal germplasm conservation of vegetatively propagated crops is now promising. The achievement of recombinant DNA biotechnology will be helpful to maintain the genetic diversity through *in vitro* DNA conservation. The benefit and limitation of *in vitro* conservation of plant tissue and genome will also be discussed.

In Situ Conservation

Maintenance of wild species or crop relatives should be in original habitats. The planned area serves as flora homeland letting boundary any set of plant community to act as an unit under influence of ecosystem and protection from human destructive impact. The advantage of *in situ* conservation benefits the population open to gene flow undergoing genetic recombination, gene exchange and mutation accumulation under ecogeographic interaction and competition influence. This permanent habitat preservation was considered as dynamic and evolutinary conservation (Frankel and Soule 1981). Even the incidence of natural selection force and evolutionary changes was subjected to slow processing but critical for creating novel genetic variability particular regarding coadaptation characters.

In order to enhance efficiency on dynamic change and future needs of genetic resources, some man-made efforts such as priority setting, selection design, population analysis, mangement and monitoring are required to be manipulated (Vaughan and Chang 1991). Vavilov indicated that the nativeland of a particular crop usually has great genetic diversity. Additional four plentiful diversity zones were added to the original Vavilov's eight centers throughout the world (Smith 1969). For global crop genetic resources, the diverse center is the best candidate for site

setting. Actual size needed for individual conservation was suggested to be hundred ha maintain thousand breeding individuals in 40 ha and extra 60 ha for natural regenerating stand (Kemp et al., 1970).

However, the woody plants, fruit trees, ornamentals and fuel trees were not included in the Vavilov centers. Recently, the information of genetic diversity and germplasm conservation in temperate fruits and nuts was well-organized by IBPGR and ISHS (Moore and Ballington, 1992). Most of related wild species and its population are rare and have scattered distribution at marginal land or mountainous area that can be assigned as nature reserve or national park to provide the last stand for survival. Tropical primary rainforest land is always rich in fruit germplasm and potential use. An investory was made of 50 ha site at Pason nature reserve in Peninsular Malaysia in which ca. 340,000 tree flora out of a total 820 species composing 76 edible fruits, which included 12 *Mangifera,* 13 *Garcinia,* 10 *Artocarpus* and 5 *Nephelium* species (Saw et al., 1991). Most of the wild species are not characterized yet, but the potential value as genetic resources is very large. Some of the species density is lower than 1 adult tree per ha behind the conservation threshold. How the dynamic change in population density, long term stability and species-specific factor or breeding behave are worth investigating. One unique geographic area, Cape of Goodhope, is occupied by fynbos flora with delicatedly shaped or attenuated leaves. The Cape area was successfully reclaimed to a nature reserve with 7750 ha maintaining 1100 species, composed of three major elements as 39 Ericoid, 29 proteoids and 52 orchids (Fig.1). This colorful ornamental habitat genebank provides a nature class and entertains people. Many of the ornamental species were bred and released to the market.

The specific wild species related to local or national crop industry are usually emphasized in priority conservation. Wild species or primitive species always possess abundant variability and include desirable characters useful for crop improvement (Hyland, 1970; Shii, 1983). Many under-exploited wild species potentially develop into new crops (Possingham, 1990). There are progressing in breeding techniques, such as overcoming sexual barrier and asexual transfer of aline gene into plants (Fig. 4). The germplasm enhancemant or direct utilization of distant species would become much feasible. Regardless of potential, the rescue and recovery of rare and endangered species are subjected to national responsibility. In Taiwan, seven nature reserves were respectively established for rare species: *Cycas formosana, Pleione taiwaniana, Kandelia*

mangrove, Keteleeria davidiana, Amentotaxus formosana and one hydrophte *Isoeles taiwanensis,* each is bounded in 50 to 100 ha protected zone (Simpson et al., 1992). The endemic species as *Malus formosana, Pyrus koehnei, Phalenopsis amible,* and some of native *Vaccinum, Actinidia, Rhododendron, Rosa* species are also in danger situation required a close protection and recovery, Westwood (1977) indicated that the *Pyrus koehnei* is resistant to fireblight, leaf spot, pear root aphid, waterlogged soil and low chilling requirement. *In situ* conservation performs as natural germplasm reactor of wild species. The positive feedback would be fruitfully.

Ex situ Conservation

The collected germplasm of perennial species departed from their original habitat is being maintained in botanic garden, experiment station, research institute and by commercial enterprises, nursery or homegarden. Historically, the botanic garden and aboreta play important role in collection and identification of wild species (Frankel and Soule, 1981), which also represent the oldest form of *ex situ* conservation in Europe back to the 15th and 16th centuries. Now, there are close to 700 botanic gardens of varying size in the world. From the 17th century in Florence Italian, a series of fruit paintings described various patterns and colors in citrus, cherry, grape, fig and others They recorded more than 30 cultivars in each crop (Fig. 2), reflecting the local people pay attention to the conservation and utilization of fruit germplasm. The Royal Kew Botanic Garden was initially established in 1759 with 5 ha, and expanded to the present 121 ha preserving more than 30,000 plant types. The botanic gardens also have made remarkable contribution to the exploration and conservation of tropical flora, and raised the development of greenhouse conservatory on modification of microclimate condition for protection of the introduced tender plants. The model of *ex situ* conservation with plant community was also simulated in some botanic gardens. The botanic gardens also actively taken part in conservation and recovery of rare and endangered species (Druy, 1974), some of the endangered species were and successfully introduced into horticultural trade.

For agricultural purpose, all the desirable characteristics related to yield, quality or resistance are worthy of exploration, and preservation in a field genebank. Herbaceous clonal lines are usually easier to maintain as seed population than the woody lines, except passion fruits or grapes

are easily duplicated and preserved by seed. Most of fruit trees, the collection of related wild and primitive species or variety might use the seeds as propagule. The germplasm of domesticates, landraces and cultivars are preferred to adopt vegetative part as preservation materials, which are propagated with selfed-roots, or grafted on the dwarf rootstock for saving space. The clonal repository serves as basic and active conservation site, ready for evaluation and utilization, and provides breeding materials. This clonal genebank is under influence of agroecosystem, we have to aware the possible high risk of field genetic erosion, and needed to protect from suffering of natural disaster and pathogen infection. The spectrum of genetic integrity is also much restricted than seed genebank. Large amounts of space and labor are required to maintain the small plant populations.

It is not feasible to preserve all the crop clonal germplasm at one repository as seed genebank. The priority is focusing on the national plant industry. The conservation network of regional repositories of fruits and nuts was well-planned in U.S.A. The NRPGR is the first of 12 proposal clonal genebanks opened in 1981 at Corvallis. It is mainly in charge of evaluation, maintenance and distribution of the national collection of pear, hazelnut, hop, mint and small fruits(Anonyous, 1981). The other national clonal germplasm repositories take part in conservation of regional major fruits were completed by 1988 (Fitzerald, 1988). The collected clonal germplasm are preserved in the field, greenhouse and other methods. The IBPGR was organized in 1974 for planning the global germplasm network and international collaboration program on basis of crop-by-crop. Comparatively, the germplasm of tuberous crops and its clonal genebanks are relatively well-established. The collected accession among potato, cassava, sweet potato and yam are recorded as 42,000, 8,000 and 10,000 respectively (Plucknett, 1987). It is a heavy load in dealing with these amounts of collected germplasm by clonal preservation. The primary genebank of potato is maintained at Huancayo in Central Peru at 3300m altitude, reduced the living accesions to 5,000 accessions, and the complementary centers are also established at the other two or three countries. Cassava genebank is established at CIAT, maintaining 4,677 accessions in 1991. One active collection of about 1,219 sweet potato accessions was preserved at AVRDC, usually regenerates twice a year with cutting (Tay et al 1987). It is estimated that the collected accessions in potato was almost covering the existing genetic integrity. But the germplasm of cassava is only included 35 % landraces and 5 % wild

species pooled in genebank (Ford-Lloyd, 1990).

The status of germplasm aquisition of fruit trees especially the tropical fruits were much limited, and varied from crop to crop. For instance, the cashew and avocado respectively have 3,600 and 2,234 accessions in field genebanks, but the *Diospyros* only have 129 accessions, the coverage percentage of landraces and wild species are not clear yet. The papaya, guava and Brazilian nut are ascribed to neglected germplasm. Basic collection of bananas are undertaken in Southeast Asia country, a total of 323 accessions are preserved at Kasetsant University of Thailand (Sylayoi and Chomchalow 1987). The banana collections at other countries including Malaysia, Vietnam, mainland China and Papua New Guinea ranged from 200 to 100 accessions. The international organization of banana and plantain germpeasm set a transit center of *Musa* germplasm at a neutral country, Belgium, routinly preserved one thousand accessions *in vitro*.

In order to ensure wide spectrum of germplasm conservation and reduce redundant accessions. Except morphological diagnosis, it is necessary to develop other genetic markers for accurate characterization of the clones (Peacock, 1984). For example, the diversification and origin between genomic groups and subgroups of edible triploid banana germplasm showed to be feasibly differentiated by biochemical markers such as isozyme *Got* genefamily as showing in Table 1, suggested that *Got* 2 gene could serve as genome marker. Both allotriploid AAB and ABB genomic groups easily differentiated due to allelic dosage effect. The Got3 could perform as subgroup marker, showed that autotriploid AAA subgroup Red, Gros Michel and Cavendish are of different genetic origin. Abundant variability in subgroup Cavendish might be derived from somatic mutation. The identification of clonal variation within subgroup required other molecular markers. It was demonstrated that DNA fingerprinting techniques could serve as a powerful tool in surveying phylogeny and discrimination of cultivars (Jarret et al., 1992, Kraemmer et al., 1992). As listing in Figure 3. The diverification of *passiflora* species could be illustrated by the RFLP of cp DNA. and evided that yellow passion fruit is differentiated from purple passion fruit (Do et al., 1992).

In vitro Conservation

The plant organ, tissue and cell are inducible to regenerate

quantatively morphological bipolarity individuals under *in vitro* conditions. The pathway of *in vitro* regeneration could be achieved following shoot-tip microcutting, axillary bud proliferation, adventitious bud formation and somatic embryogenesis, depending on explant attributes and culture medium. Both organogenesis and somatic embryogenesis are based on serial cell duplication and differentiation. All the regenerants come from cloning are identical to the mother stock. Therefore, small pieces of explant could represent the germplasm used as clonal storage material. Morel (1975) suggested that meristem culture technique can apply to the long-term storage of cultivated plants. Dale (1976) calculated that 800 cultivars of grape could be stored *in vitro* in a few square meters of space instead of one hectar field genebank. The controlled *in vitro* storage could prevent the erosion as fields suffer from natural catastrophe or disease infection. It is also helpful in maintaining and multipling specific genotypes such as sexual infertility, male sterility and self-incompatible or haploid lines. The test tube plantlets serve as safe bridge for transport and international germplasm exchange.

There are three major aspects when considering tissue culture for genetic conservation: 1. Genetic stability should be maintained. The somaclonal variation might be encountered due to perexisting and induced mutation or activated transposition (Scrowcroft and Larkin, 1982). Choosing shoot-tip as storage material, utilizing multiple shoot proliferation, will reduce the subculture generations and given minimal medium, all these manipulations are helpful to secure the stability. The testing of genetic purity is necessary for ensuring trueness-to-type of each accession in reliable maintenance. 2. Absence of disease must be guaranted, the target accession should pass through through healthy diagnosis. The initial eradication could be obtained by apical meristem culture or micrografting method. During storage period, air carrying infection such as mite and mold might destroy the accessions. Giving filtered aeration, low humidity and cold room temperature are useful. 3. The regenerability retention, the regeneration pathway and capability might vary with cultivars, tissue and medium. Some woody plants are difficult to regenerate. We have to overcome the problem through *in vivo* or *in vitro* rejuvenation treatment (Pierick, 1990; Hammatt and Grant, 1993), or choose high organogenic or embryogenic tissue as preserving materials. The storage period is actually based on the explant and plantlet longevity of regeneration potential *in vitro.*

In vitro conservation could be optionally conducted with slow

growth and cryogenic system for working collection and basic collection respectively (Roca et al., 1983). The slow growth method is to culture the shoot tip or bud under modifying condition. Let the plant alive but retarded growth processes through physical or chemical restriction. Longer storage can be subcultured at intervals. This storage method has considerable advantage in that the stored material is readily available for use, can be easily visible to be alive, and accession is rapidly to be duplicated when it is required. Various efforts on nutrient, osmotic stress, gas component, carbon sources, retardants and environmental factors have been imposed to restrict the growth (Bajaj, 1979; Withers, 1980). An alternation of the physical conditions most often by a reduction of the temperature, the cassava, sweet potato, coffee and lilies, given 15-20 ℃ temperature with minimal medium, one stored growth cycle could be extended to 12-18 months. The potato, strawberry, pear and apples can be stored at 0-6 ℃ as long as 2-3 years for one generation. Sometimes inhibitors such as CCC, ABA are used. Mix (1984) used nodal explants of 220 potato cultivars as starting material for long-term storage. Plantlets were maintained at 10 ℃ in the presence of N-dimethyl-aminosuccinamid acid. The adventitious buds of banana formed at MS medium containing higher agar concentration developed into corm-like structure, which were close to dormant state for one year still remaining alive, and rapidly sprout when decreased agar concentration or in liquid medium (Ma and Shii, 1974).

Slow growth storage of germplasm become a routine technique in conservation of most tuberous crops. Over 1300 cassava accession at CIAT germplasm center have been transformed into *in vitro* conservation. 6000 clonal accessions can be accomodaled in 5x6x3m storage room, using 5 vessels of 5x14 cm per accession (Roca et al., 1983). The INIBAP organized a transit center for *in vitro* storage and exchange of *Musa* germplasm in Leuven, Belgium. The shoot tip of each accession was multiplied and stored under 15 ℃ and 2,000 Lux lighting condition according procedures developed by Baneejee and Langhe (1985). For healthy diagnosis and field evaluation, parts of each clone were send to CIRAD (France), QDPI (Australia) and Philippines. As soon as the transit center receives negative indexing, each accession enters the medium term storage at a number of 20 cultures, a total of 1032 accession are preserved for research and germplasm exchange (DeSmet and Van den Houwen, 1991).

Cryopreservation is based on the reduction and subsequent arrest of metabolic function by the reduction in temperature of materials at ultra

-low temperature while maintaining viability. Bajaj (1977) showed the prospects of cryopreservation for the establishment of germplasm banks, which has numerous advantages. The propagule can be stored, with no need to subculture; retain morphogenetic potential and prevent aging; save space and manpower. Basic efforts of cryopreservation need to enhance the propagule tolerance in freezing process, and prevent the occurrence of ice crystallization and recrystallization during cooling and thawing, which do mechanical damage to the cells (Nitzache, 1983). Preacclimation, cryopotectants and prefreezing are amenable to minimize the incidence of intracellular ice formation, increase the stability of plasma membrane and decreased the lowering nucleation temperature at -42 ℃ . The nucleation temperature of nonacclimation is -15 ℃ (Steponkus, 1985).

The type of preserving materials such as shoot tip, apical meristem or embryos are considered the most desired propagules for germplasm conservation regarding genetic stability, but the survival percentage is often 60 % or lower (Kartha, 1985; Towill, 1988). Pregrowth cool acclimation or encapsulation-desiccation system have been applied to improve the cryogenic tolerance. The more cell homogeneity in the propagule, such apical meristem, as callus or cells, would have the higher viability under ultra-low temperature condition (Henshaw, 1984). But the more complex part is to regenerate after thawing and risk of somaclonal variation. Protopalsts of *Atropa, Nicotiana* and *Datura* subjected to -196 ℃ storage in presence of DMSO, mannitol and sucrose cryoprotectants, after 35 ℃ thawing, the former two obtained 71 to 53 % regeneration, even the somatic hybrid protoplasts still achieved 10-18 % survival (Bajaj, 1989). Predessiccation with vitrification solution containing ethylene glycol, PEG, DMSO and sorbitol that have been shown to enhance the cryogenic survival of various types of stored materials (Sakai et al., 1990; Towill, 1990; Langis and Steponkus, 1990).

The factors of pregrowth condition, cryoprotectant formulation, cooling rate and thawing temperature have greatly affected the survival, which are needed to be defined for different crops. Withers (1984) recommended the cryopreservation procedures as follows: for cell propagule, pregrow on standard medium or medium supplemented with 6 % mannitol for 4 to 7 days. Then harvest during active growth cryoprotected with 0.5M DMSO, 0.5M glycerol and 1M sucrose or proline. Dispense into ampoules freezon at 1 ℃ /min to -35 ℃ ; thereafter, hold at -35 ℃ for 40 min. then transfer to liquid nitrogen. These stored cells thaw rapidly in warm water at 40 ℃ and recover on semi-solid medium. The procedures

for protoplast are very similar to the above recommendation; or substitute the cryoprotectant with 5 % DMSO added with 10 % glucose.

DNA *in vitro* Conservation

Major plant genomes are localized in the nucleus, and two other small genomes exist in the chloroplast and mitochondria. Interms of DNA content, the nuclear genome size varies from species to species, such as soybean and wheat are respectively calculated as 8.69×10^8 b.p. and 1.5×10^{10} b.p. DNA, and close to 0.9 and 16.5 pg per haploid cell (Benett and Smith, 1976; Roger and Bendich, 1988). The genetic informations are loading in chromosomal DNA, although the DNA content not all means to be functional sequences but substantial constant with specific species. The genetic diversity can be elucidated by the degree of DNA homology. Therefore, the collected and reserved genomic DNA is capable to represent as genetic resources.

Plant DNA can be isolated and purified routinely (Murray and Thompson 1980, Watson and Thompson, 1988). The DNA yield changes with crop species and tissue types, the leaf samples usually have high yield average 14-49 μ g/mg tissue. It is only need 55mg of tissue to obtain 2.9 μ g DNA, that is enough for detecting the single copy gene (Roger and Benedich, 1988). Even a few copies of isolated DNA, that can be amplified rapidly through polymerase chain reaction *in vitro.* It was demonstrated that the mummified plant tissue is still extractable to obtain DNA (Roger and Benedich, 1985). It turned out, the freeze-drying tissues can be stored and serve as delaying DNA conservation, considered potentially to save endangered germplasm, and could be used whenever we needed (Adams, 1992). The purified DNA with nuclease-free is able to be preserved in alcohol solution indefinitely. Theoretically, the genetic resources of all the global flora DNA are feasible to be maintained in one house (Ford-Lloyd, 1990). The DNA library could be also achieveable by in vivo deposition and amplification in prokaryote cells.

Utilization of DNA genebank could be approached by genetic engineering. The isolated genes could be optionally delivered into target cells through vector, electroporation, particle bombardment or direct uptake and pollen-tube pathway. *In vitro* culture of the transformed cells regenerate into plantlets via organogenesis or embryogenesis, so called transgenic plants. A few of the desirable sequences related to resistance of disease, insect, herbicide, drug or cold stress, and the genes involved in

developmental regulation of storage proteins, pigmentation and fertility are already available, and successfully transformed and expressed in the transgenic plants (Fisk and Dandeker, 1993). The progress in biotechnology would be delightful for the use of conserved DNA germplasm, to improve the crop without limitation by phylogeny relationship and sexuality. The introduction of foreign genes do not need to change the genetic background of the breeding materials. This molecular breeding is highly potential for improvement of vegetatively propagated crops, especially to the sterile crops, such as bananas. The transformed cells developed into somatic embryos and positive expression is showing in Figure 4. (Liao et al., 1992).

The development of nake DNA bank for conservation of the genetic resources reveals as the most economical way. In consideration of molecular biology in utilization of conserved DNA, the progress in genomic sequencing and mapping is still very limited. Most of the genes involved in quantitative traits are not well-identified yet, and the engineered sizes of foreign genes restricted to a few copies. However, if the diverse genome or the DNA sequences could be preserved particularly for the extinction threatened or endangered species, it would serve to make more readily available and expanded the breadth of genetic resources whenever required, and to include those of the past (Ford-Lloyd, 1990).

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Fig. 1 *In situ* conservation of fynbos flora in nature reserve at Cape of Goodhope.

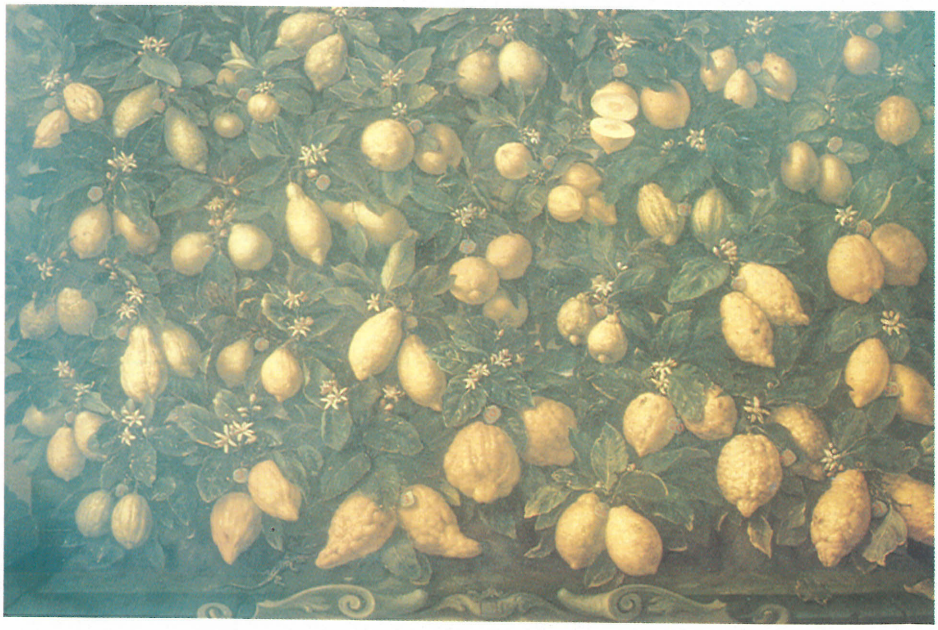

Fig. 2 Utilization of genetic diversity in fruit industry by ancient people depicted by paintings.

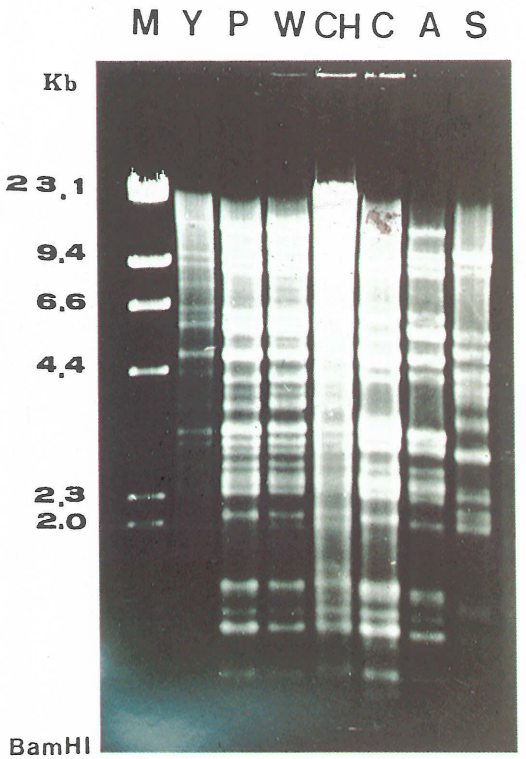

Fig. 3 Characterization of *Passiflora* species by cpDNA fingerprinting through Hind III digestion. Y: *P. edulis* f. *flavicarpa*, P: *P. edulis*. W: wild escape of *P. edulis*; CH: *P. coccinea* x Y; C: *P. alata*, S: *P. suberosa*.

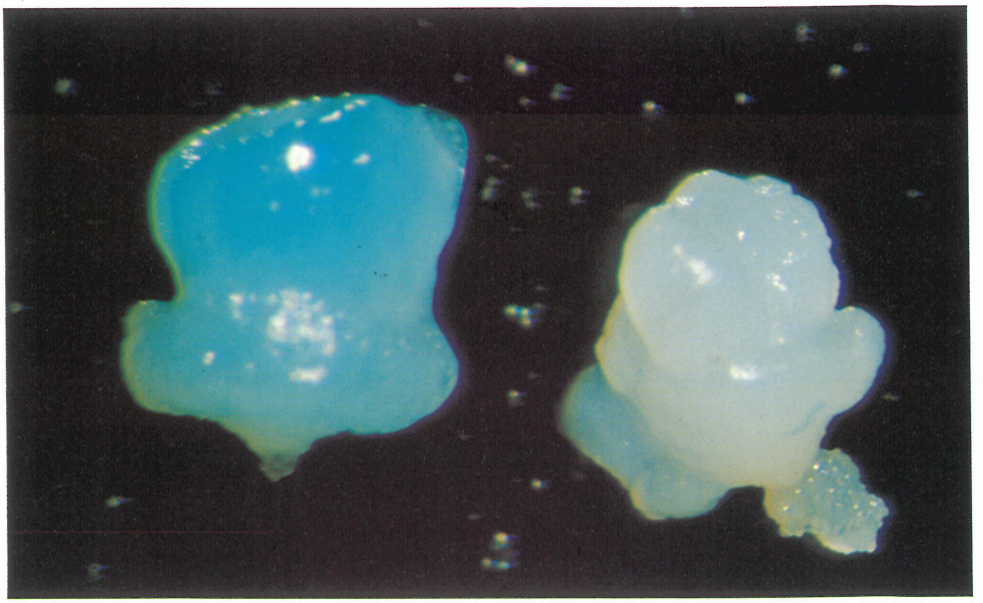

Fig. 4 The asexual trausfer of foreign DNA transformed into triploid banana culture cells and regenerated into transgenically somatic embryos (left one)

Table. 1 Characterization of germplasm diversity through Got genefamily markers on the introduced polyploid banana clones.

	genotypes and genomic groups			
Got 2 / Got 3	*FFF* AAA	*FFS* AAB	*FSS* ABB	*SSS* BBB
genotypes and subgroups	*aah* Red (5) *bbh* Gros Michel (7) *bhh* SH3436 (3) *bch* Cavendish (57) — — AAAA *FFFF* IC_2(4) Bodles altfort (1)	*bbh* Raja (8) *bch* Kanaba (5) *bhj* Kabok (15) *cch* 3-6(2) *dhh* 3-1(1) *hhh* Laknau(3) *hhj* Corne plantain	*bhh* K. hakmuk (2) *bjj* Apple (2) *hhh* P. bayu (4) *hhj* P. awak (5) *hjj*	*hhh* Malaca (2) — — ABV FSF_2 Tibok (1) — — BVX FF_2S_1 Pitogo (4)

Note: 1. In parenthesis represents the number of clones. The accessions of SH3436 and AAAA genomic group are man-bred clones.

2. The *Got2* genotypes of wild species *Musa acuminata* (AA) and *M. balbisiana* were assessed as *Got2-FF* and *Got2-SS* respectively.

3. All of the alternative *Got3* alleles present in these triploids are also detectable in wild species. *Got3-a, b, c, d* types are specific to A genome. *Got 3-h* is common in both of A and B genomes. *Got3-J* is specific to B genome.